THE EFFECTS OF SEX AND AGE ON VACCINE-INDUCED IMMUNITY AND PROTECTION

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

April 2018

ABSTRACT

Background: Immune responses to vaccines decline with age, but whether this immune senescence occurs similarly for males and females is rarely considered in either preclinical or clinical studies of vaccines. Using both human serum samples and a mouse model of influenza vaccination, this project aims to understand the interaction between sex and age on vaccine-induced immunity and protection against influenza virus challenge. I hypothesized that among both humans and mice, females of reproductive ages would develop higher antibody responses, which in mice would result in better protection upon challenge as compared to young adult males. Further, among aged individuals, sex differences would either wane or be reversed because of the lack of impact of reproductive hormones on immune responses.

Methods: To test my hypothesis, I used human serum samples collected prior to and after receipt of an inactivated, monovalent A/Cal/09 H1N1 vaccine, and measured cytokine, neutralizing antibody, and cross-neutralizing antibody responses in young adults (18-41 years of age) and aged adults (65+ years of age). I also compared their neutralizing antibody responses with their hemagglutinin inhibition (HAI) antibody titers. Further, using a mouse model, I vaccinated young (9-10 weeks) and aged (68+ weeks) mice with an inactivated A/Cal/09 H1N1 virus to evaluate their vaccine-induced antibody responses, including HAI, neutralizing antibody, IgG, IgG isotypes, IgG avidity, and cross-neutralizing antibody titers. To correlate antibody responses with protection, we challenged the mice to an H1N1-drift variant virus and monitored their morbidity and mortality.

Results: From the human serum analysis, young adult females developed higher IL-6, neutralizing antibody responses against A/Cal/09, and to a lesser extent against A/Mich/15 H1N1, than either young adult males or aged adult females. Overall, aged adults had lower neutralizing antibody responses than young adults. In contrast, neither age nor sex differences in HAI titers were observed, in which all individuals had HAI titers that were more than 4-fold higher post- as compared with pre-vaccination. Consistent with human data, young female mice developed higher vaccine-induced neutralizing antibody, and to a lesser extent cross-neutralizing, responses and were better-protected following influenza virus challenge than either young male or aged female mice. Young adult female mice also had greater IgG class switch recombination and antibody avidity than either young adult male or aged female mice. No sex differences in antibody titers, antibody quality, or morbidity post challenge were observed in aged animals.

Conclusions: Taken together, we show robust sex differences among young adults in influenza vaccine responses but such sex differences are not observed among aged adults, pointing to sex-specific changes during immunosenescence.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Sabra Klein for her continuous guidance and support during my graduate study. Thank you for your wise counsel and for being a wonderful mentor. I would like to thank Dr. Ashley Fink for her advice, patience and for teaching me all the techniques that went into this thesis. I would also like to thank my lab mates Landon vom Steeg, Kyrra Engle, Dr. Meghan Vermillion and Rebecca Ursin for their encouragement, insightful feedback and stimulating discussions. Thank you to Dr. Andrew Pekosz and his lab for their collaboration and feedback. I would also like to thank my secondary reader Dr. Kawsar Talaat for her input and advice. I also thank my friends in the school for their incredible support both personally and professionally. I thank my extended family for welcoming me and helping me get through graduate school- Nikitha, Krishna, Shali, Sowmya, Pradeep, Mohan, Hanisha and Jyoti. Last but not least, I thank my parents and my grandmother for being my rock and their unconditional love and support in everything I do.

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INTRODUCTION

Influenza A viruses are negative sense RNA viruses that are responsible for respiratory tract infections causing significant morbidity and mortality due to seasonal epidemics, occasional pandemics and regional outbreaks. Due to a lack of proofreading mechanisms during replication, these viruses accumulate mutations and exist as quasispecies (Hannoun 2013). Antigenic shift occurs as a result of reassortment of gene segments from zoonotic reservoirs into current circulating strains while antigenic drift occurs through the interactions between influenza viruses and the host immune responses (Webster and Govorkova 2014). Infections can be prevented by administration of yearly influenza vaccinations that can induce sufficient antibody responses against current circulating strains. However, despite the recent advances in vaccine technology, current influenza vaccines provide inadequate protection (10-60% effective) against seasonal influenza A viruses and little or no protection against pandemic strains (Paules et al. 2017). This efficacy is lower in aged populations (individuals >65 years), increasing their risk of hospitalizations from acute respiratory and cardiovascular illnesses (McElhaney and Dutz 2008).

A decline in functioning by multiple components of humoral and cell mediated immunity during aging poses a need for strategies and interventions to improve the efficacy of influenza immunizations. Apart from aging, biological sex and gender-specific behavior can also influence vaccine uptake and efficacy. Prior to menopause, a number of studies suggest that females of reproductive ages show greater influenza vaccine efficacy and experience higher adverse effects from vaccinations (KatieL.Flanagan 2017). However, most studies do not disaggregate and analyze differences between the sexes and even fewer ones consider how sex and age interact to alter vaccine efficacy (Engler et al. 2008). Therefore, the aim of this study is to incorporate sex and age as biological variables to comprehend their effect on influenza vaccine responses in both humans and animals.

Aging and Innate Immunity

Evolutionary biologists define aging as a progressive, time-related decline in physiological functioning and the most prominent feature of aging is the gradual decline in immune function (immunosenescence) (Flatt 2012). The innate immune system is a germ-line encoded, first line of defense against invading pathogens. Comprising of anatomical barriers, phagocytic cells, complement system, cytokines and antigen presenting cells, this arm of the immune system is non-specific, and initiates adaptive immune responses (Medzhitov and Janeway Jr 2000). Senescence in the innate immune system is typified by reduced first-line defense against a pathogen, decreased ability to initiate adaptive immunity and a chronic inflammatory state (Boraschi et al. 2013). This inability is compounded by a reduction in the proliferative capacity of hematopoietic stem cells (HSCs) with age (Dykstra and de Haan 2008). The kinetics of this reduction, however, vary widely within a population, strongly indicating an underlying genetic component. This hypothesis is further bolstered by a review of murine studies across different strains. C57Bl/6 mice show an increase in HSC numbers with age but a decrease is observed in CBA, DBA/2 and Balb/c strains (Dykstra and de Haan 2008). Regardless of the count, these aged HSCs accumulate deficiencies over time and are less robust in both humans and mice. Furthermore, differentiation of aged HSCs is skewed towards myeloid lineage, thus upsetting the balance of lymphoid to myeloid cells in the system (Chen 2004, Dykstra and de Haan 2008). Such changes during the production of innate immune cells could have a significant impact on recognition and effector activity against microbes.

Recognition of highly conserved patterns by the immune system is facilitated by pattern recognition receptors (PRRs). A key member of this family of receptors is the toll-like receptor group (TLRs) which are present on many cell types, including antigen presenting cells (APCs). Elderly populations show a reduced expression of these key receptors on dendritic cells (DCs)

and a subsequent alteration in the downstream signaling pathways upon its activation (Panda et al. 2010). Such age-associated deficiencies post immunization affect TLR-induced cytokine production and seroconversion to the vaccine strains (Panda et al. 2010).

Apart from defective TLR signaling, aged DCs are profoundly impacted in their ability to prime adaptive immune cells. A reduction in PI3K signaling, especially in myeloid DCs, reduces their ability to activate naïve CD4+ T cells (Agrawal and Gupta 2011, Solana et al. 2012). Downstream effects, typically, lead to increased NF-κB signaling, contributing to a chronic pro-inflammatory state commonly seen in the elderly (Solana et al. 2012). Furthermore, antigen presentation is another key function of DCs that is altered by inefficient phagocytosis with aging (Agrawal et al. 2007). Such a decrease in phagocytic activity is synonymous to other innate immune cells as well. Studies show macrophages, monocytes, polymorphonuclear neutrophils show considerable reduction in phagocytosis, chemotaxis and reactive oxygen species production- all functions that are essential to activate T cells (Solana, Pawelec, and Tarazona 2006, Van Duin et al. 2007, Chatta et al. 1993, Niwa et al. 1989, Wenisch et al. 2000, Fulop et al. 2004, Fortin et al. 2008, Butcher et al. 2001). APCs are required to detect, process, present the vaccine antigen to activate T cells and confer protective immunity (Boraschi et al. 2013). A comparative study in human skin, shows a decrease in Langerhans cells with sun exposure and age (i.e., photoaging) and another observed reduced accessory function in alveolar macrophages in aged humans (Toyoda and Bhawan 1997, Zissel, Schlaak, and Müller-Quernheim 1999). These age-related changes in innate immune cells may pose significant challenges to vaccine success.

A prominent characteristic of aging is the chronic, low-grade inflammation despite the absence of an infection. C-reactive protein, IL-6 and other cachexic cytokines are found in higher concentrations with aging and are commonly associated with diabetes or cardiovascular diseases. Meanwhile, aged individuals who show few age-related co-morbidities (healthy aging) maintain a balance of pro- and anti- inflammatory profiles in their system, thereby reducing inflammation

associated morbidities (Franceschi and Campisi 2014, Franceschi, Bonafè, et al. 2000, Franceschi, Valensin, et al. 2000). Commonly termed "inflammaging", the exact etiology of this inflammation, however, is still to be understood. Some speculate the accumulation of danger-associated molecular patterns (DAMPs) with age, while others attribute it to alterations in one's microbiota and their metabolic products with aging. Accumulation of pro-inflammatory secretions by senescent cells and imbalances in coagulation pathways are also surmised to augment inflammaging (Franceschi and Campisi 2014, Rodier and Campisi 2011, Franceschi, Valensin, et al. 2000, Tchkonia et al. 2010, Biagi et al. 2011, Salminen, Kaarniranta, and Kauppinen 2012). Aging of the innate immune system is a combinatorial effect of disparities in the production, effector and maintenance pathways of the cells involved and the associated signaling molecules.

Aging and Adaptive Immunity:

An adaptive immune response, unlike innate responses, involves the genetic rearrangement of receptors and development of responses that are highly specific to a pathogen (or antigen) (Alberts et al. 2002). Comprised primarily of B and T lymphocytes, the adaptive immune system is also capable of generating a swift memory response upon secondary exposure, which is the principle of vaccination.

B lymphocytes are the principal mediators of humoral immunity, producing antibodies that bind to antigenic sites of a pathogen. Senescence of humoral immunity is observed both in terms of the quantity and quality of responses. The pool of early B-cell progenitors is reduced with age, affecting the generation of pro and pre-B cells required for a B-cell lineage commitment (Cancro et al. 2009, Miller and Allman 2003). Correspondingly, the pool of switched memory B cells (that produce either IgA or IgG) decreases with age while the percentage of IgM memory cells remain unaffected (Frasca and Blomberg 2011). Aged individuals were unable to increase

their pools of switched memory B cells post influenza vaccinations, mounting insufficient antibody responses (Frasca et al. 2010).

The expression of several transcription factors such as E2A and RAG, are crucial for B cell development, and diminish with aging (Labrie et al. 2004, Riley, Blomberg, and Frasca 2005). Competent bone marrow stromal cells can provide support to developing B-cells through chemical signals such as IL-7 (Cancro et al. 2009). Alterations in the senescent microenvironment of bone marrow add to the intrinsic deficiencies of developing B cells, creating a "snowball effect" on the production and diversity of B-lymphocytes (Cancro et al. 2009). Furthermore, the presence of Age-associated B cells (ABCs), a heterogeneous subset associated with aging, produce high levels of pro-inflammatory cytokines that can induce apoptosis in pro-B cells (Riley, Khomtchouk, and Blomberg 2017). Accumulation of ABCs and memory subsets along with a reduced lymphopoiesis greatly limits antibody diversity and increases the production of autoreactive serum antibodies with aging (Weiskopf, Weinberger, and Grubeck-Loebenstein 2009). Reduced B cell diversity and an inability to produce sufficient naïve populations likely affects vaccine responses and its efficacy with aging (Scholz et al. 2013).

During an immune response, antibodies (or immunoglobulins) are generated to neutralize pathogens and aid in their clearance (Alberts et al. 2002). Different classes of antibodies can be produced by B-cell class switching which then execute specific functions, working in tandem to create an effective response to an antigen. Moreover, antibodies can undergo somatic hypermutations in germinal centers that enhance their affinity for the antigen. Aged individuals, however, can fail to undergo this vital class switching and affinity maturation, primarily due shortcomings in class switch recombinase (CSR) and activation-induced cytidine deaminase (AID) expression (Frasca et al. 2011). Individuals with CSR and AID defects are prone to increased opportunistic bacterial and viral infections (Frasca et al. 2011). Several influenza vaccine studies substantiate decreased production of IgG and IgA in elderly compared to young

adults (Murasko et al. 2002, Sasaki et al. 2011). A study measuring the kinetics of AID expression demonstrated a strong positive correlation between AID mRNA expression and neutralizing antibody titers post seasonal influenza vaccination (Frasca et al. 2010). This age-related decrease in AID expression has potential implications in the production of secretory IgA and the maintenance of immune responses at mucosal surfaces in respiratory, intestinal and urogenital tracts (Holmgren and Czerkinsky 2005). Gut associated lymphoreticular tissues (GALT), nasopharyngeal-associated lymphoreticular tissues (NALT), IgA responses in the gut have been reported to reduce with age. Changes in microbiota (reduction in protective anaerobes) could be a consequence of impaired IgA production in the elderly (Cancro et al. 2009, Fujihashi and Kiyono 2009).

Overall, the failure of the aged immune system to maintain B cell repertoires adding to their inability to produce new, robust B cells reduce their immune reactivity and reduce novel pathogenic defense and vaccine efficacy.

T lymphocytes make up the cell-mediated arm of the adaptive immune system. These lymphocytes are activated upon antigen presentation by APCs and differentiate into functional subsets working collectively to eliminate a pathogen. Helper CD4+ T cells (Th) interact with other immune cells to stimulate and coordinate responses while cytotoxic CD8+ T cells (Tc) actively seek out and kill infected cells. Different classes of pathogens, including viruses, extracellular bacteria and parasites elicit different CD4+ T cell effector responses (Th1, Th2, Th17 primarily) that are crucial for pathogen clearance and subsequent memory response (Alberts et al. 2002). Immunosenescence can shift the balance of these effector CD4+ T cell responses, increasing the risk of opportunistic infections. Immune aging is generally associated with a Th1-to-Th2 shift, due to a defective polarization, cytokine changes and intrinsic changes within T cells. The decline of Th1-type responses adversely affects the ability of T cells to control viral infections and tumors (Maue et al. 2009, Shearer 1997). The recruitment of cells to active sites of

infection is also dependent on the balance of Th1 vs Th2 responses. CD8+ cytotoxic T cell (Tc) cell activity in human influenza studies show their recruitment to lungs is dependent upon Th1 cytokine production (McElhaney et al. 2006). Aged individuals are unable to clear lung infections effectively due to a reduced IFNγ:IL-10 production rendering them unable to recruit CD8+ T cells effectively (McElhaney et al. 2006).

The effect of aging on the immune system is most prominent in cellular immunity, acutely affecting T cell production, maintenance and function. The generation of naïve CD4 and CD8 T cells commences in the bone marrow and these double negative cells mature into single positive T cells in the thymus. The thymus provides a specialized microenvironment for the thymocytes to develop during early years but proceeds into events of rapid regression with aging (Aw, Silva, and Palmer 2007). This thymic involution is hypothesized to be mediated through hormonal and cytokine changes, progressively decreasing thymic output with age (Hakim and Gress 2007). Along with hampered production, aged individuals show reduced T-cell receptor signaling arising from impaired Ca2+, NFκB, NFAT and tyrosine phosphate pathways (Grossmann et al. 1991). Such signaling defects can lead to impaired cytokine production, commonly observed in aged T cells (Haynes and Swain 2006). Reduced cytokine production has further consequences downstream by affecting clonal expansion, activation of other immune cells and contributing to inflammaging as T cells are a key source of cytokine production (Haynes and Eaton 2005). *In vitro* studies with aged helper T cells reveal reduced TCR responsiveness and reduced synaptic cleft formation. Such intrinsic functional defects in CD4+ helper T cells accumulate with aging and can affect CD8+ T cell responses as well (Haynes and Swain 2006, Weng 2006, Haynes and Maue 2009). Helper T cells are more susceptible to apoptosis on aged microenvironment, while CD8+ Tc cells develop apoptotic resistance, shifting the balance of these subsets (Targonski, Jacobson, and Poland 2007). This imbalance is also reflected through the preference of CD8+ T cells to certain epitopes but not the others. In mice, the TCR repertoire

from aged mice is only a subsection of the repertoire generated in young mice, reducing the ability of aged mice to generate responses against immunodominant epitopes, i.e., characterized by nucleoprotein (NP) epitope of the influenza A virus (Yager et al. 2008).

The accumulation of T memory cells (CD28 loss) under steady state conditions can be an indicator of adaptive immune senescence. Studies analyzing T cell populations in healthy elderly individuals reveal a distinct population of T cells that balance their CD28+ and CD28- subgroups comparable to young individuals. The frail (or institutionalized) elderly meanwhile, showed a near complete loss of CD28+ subsets (Saule et al. 2006, Naumova et al. 2004). Moreover, aged memory cells are limited in their capacity to respond to chemokines (e.g., CCR7) and costimulatory molecules (Saule et al. 2006). Terminally differentiated T cells are persistent T cells that have lost costimulatory receptor CD27 and CD28 are a significant contributor to aging immune system and other age-related co-morbidities (Pawelec et al. 2005).

The two arms of the adaptive immune system- humoral and cell mediated immunity are in continual communication with each other to coordinate an effective immune response. Germinal center (GC) formations are crucial for such B-T cell interactions. B cells can present antigens to T cells, activating cell mediated responses, while helper T cells stimulate B cells to undergo class switching, somatic hypermutation producing higher avidity antibody (Alberts et al. 2002). *In vivo* studies using aged mice have shown a progressive decline in such germinal center responses, mainly due to defective transportation of antigens and subsequent downstream effects (Zheng et al. 1997). Several co-stimulatory molecules that mediate T-B cell interactions such as CD28, CD40, CD80, CD86 do not elicit desired results, producing a comprehensive hyporesponsive state in the aged T cells (Sasaki et al. 2011, Zheng et al. 1997). Studies also show a reduced immunosynapse formation in aged T cells due to an inherent inability to move substrates into synaptic regions (Haynes and Eaton 2005). Furthermore, impaired germinal center formation in aged individuals leads to an inability to perform class switching and affinity

maturation, leading to ineffective antibody formation (Zheng et al. 1997). Altogether, thymic involution, accumulation of intrinsic defects and shifts in T cell subpopulations with aging lead to profound changes, rendering them incapable of mounting an efficient immune response.

Sex and immunity:

Sex refers to a biological difference between males and females while gender is a sociocultural construct of males and females (Oertelt-Prigione 2012). Sex can influence gender roles (e.g. testosterone increasing aggressive behaviour) and gender roles can affect biological processes (e.g. differential exposure to chemicals leading to genetic or epigenetic changes) (Oertelt-Prigione and Regitz-Zagrosek 2011). Nevertheless, this study focuses on biological differences and hence discusses sex differences in immune responses between males and females.

Sex steroid hormones, X-chromosome genes and the microbiota are considered the primary mediators leading to sex-biases in the immune system (Klein and Flanagan 2016). Estrogens and androgens bind to their respective receptors on immune cells, leading to downstream signaling events to modulate immune responses to microbes or vaccines (Fish 2008). Generally, estrogen at physiological concentrations can facilitate pro-inflammatory responses while testosterone and progesterone are generally anti-inflammatory. However, these hormonal modulations are more complex and nuanced as often described by previously (Oertelt-Prigione 2012).

Moreover, it is important to note age interacts with sex to alter immune responses. Sex hormones and gene expression patterns undergo considerable alterations throughout the life course. Although sex differences are seen prior to puberty and post menopause, sex biases in immune system are most prominent when females are of reproductive ages and sex steroids can exert their maximal influence. A population level epidemiologic analysis was performed to understand the role of sex, gender, and age on infectious disease incidence in ~0.5 million

infectious disease cases in Brazil. The results indicated a primary role for physiological mechanisms (e.g., hormonal influences and X chromosome inactivation) while gender-specific behaviour played a secondary role in male-female biases in infectious disease susceptibility. More importantly, their study stands to caution against the extension of biomedical studies across individuals of different ages and sex and signifies the need for an "age and sex stratification" to better understand infectious disease pathogenesis (Guerra-Silveira and Abad-Franch 2013).

Sex Differences in Immune Responses:

Post Puberty/Adulthood:

Estrogen receptors (ERs) are expressed on macrophages, DCs, natural killer cells and other innate immune cells and their activation can augment or inhibit immune signaling pathways. Notably, estradiol signaling in female plasmacytoid DCs or PBMCs significantly increases type 1 IFN production in viral pathogenesis (Meier et al. 2009, Berghöfer et al. 2006). Other cytokines and chemokines such as IL-6, IL-23, IL-12 and IL-1β are also augmented in innate immune cells from females after stimulation with TLR ligands, plausibly via sex steroids' receptor activity (Kovats 2015, Traub et al. 2012). On the other hand, progesterone suppresses TLR mediated cytokine production, particularly IL-6 and IL-12 in DC subsets (Hughes and Clark 2007). High-dose of estrogen has immunosuppressive effects by promoting the production of IL-10 and tolerogenic DC subsets (Hughes and Clark 2007). Estrogen can also influence migration of various innate immune cells by promoting the secretion of tissue homing chemokines like CCL 19/MIP3β (Hughes and Clark 2007). Testosterone, meanwhile, increases anti-inflammatory responses by promoting IL-10 production and inhibiting IFNγ secretion (Pestka et al. 2004, Lotter et al. 2013). Male PBMCs produce higher IL-10 and lower IFNγ upon TLR 7,8 or 9 stimulation by their respective ligands or viruses (Torcia et al. 2012). Such differences in cytokine and chemokine productions can influence macrophage polarity. Macrophages from male

mice undergo M1 polarization (i.e., pro-inflammatory) while female macrophages undergo M2 polarization (i.e., anti-inflammatory) upon Coxsackievirus infection. This shift leads to myocarditis in male mice but protects female mice (Li et al. 2009). However, biases in macrophage polarizations can predispose females to asthma and other Th2- type hypersensitivity disorders as well (Jaillon, Berthenet, and Garlanda 2017, Warren et al. 2017)

Escape of certain immune genes from X chromosome inactivation (XCI) can lead to differential expression patterns of TLRs and other pattern recognition receptors in males and females. The patterns of XCI in human tissues demonstrated these sex-specific gene expressions across multiple tissues, several of which are immune system associated (Tukiainen et al. 2016). Murine bone marrow derived macrophages have higher TLR7 and TLR8 expression in females compared to males (McDonald et al. 2015). *Tlr8* expression is seen from both active and inactive X chromosomes, leading to its over-expression in females. This overexpression has also been attributed to increased IFNα production by DCs from females and could be a critical factor in pathogenesis of autoimmune diseases such as systemic lupus erythematosus (Berghöfer et al. 2006). Apart from increased cytokine production, neutrophils from female rats show increased phagocytic activity and higher nitric oxide production post LPS stimulation (Spitzer 1999). Additionally, such sex-specific biases in macrophages and neutrophils are also observed in responses following injury by trauma or infection (Bird, Karavitis, and Kovacs 2008). Females patients show higher proliferation of immune cells and greater IL-6 production after burn injury (Gregory et al. 2000). These differences could also potentially mediate adverse events following vaccination, where females are more likely to report pain and local reactions at the injection site than males (Cook 2008). Antigen presentation is another important aspect of innate immunity that has also been shown to be influenced by sex. Female APCs are more efficient than male APCs in inducing secondary immune responses in primed lymphocytes (Weinstein, Ran, and Segal 1984). APCs can be modulated by sex steroid hormones and females are more efficient than males at

presenting antigens to subsequently induce Th1-type adaptive immunity (Weinstein, Ran, and Segal 1984, Wilcoxen et al. 2000).

Consequently, an increased Th1 response in females provides better protection to viral infections but predisposes them to hypersensitivity disorders. A plausible explanation for Th1 bias in females is due to increased dehydroepiandesterone (DHEA) production. This upregulation can be attributed to escape of XCI by steroid sulphatases, thereby increasing DHEA production in macrophages of lymphoid tissues (Reza 2009). Furthermore, PPARγ (peroxisome proliferator-activated receptor gamma), a regulator of lipid metabolism shows sex-specific regulation of Th1, Th2, Th17 differentiation (Park et al. 2016). PPARγ selectively inhibits male Th17 differentiation and PPARγ agonists inhibit germinal center formation selectively in females (Park et al. 2016). Such sex-specific T cell ligands and their responses can pave the path for better treatments for autoimmune diseases. Apart from differences in T cell polarizations, female CD8+ T effector cells are more effective and cytotoxic than male CD8+ T cells (Sterling et al. 2005). This manifests as a sex bias in HIV infected individuals where females show a slower progression to AIDS than males with lower viral RNA presence and higher CD4+ T cell counts (Siddiqui et al. 2009). Alternatively, females also develop higher alloreactive CD8 T cells after solid organ transplantation, increasing their risk for acute allograft rejection (Mifsud et al. 2008). Treg cell functions are also influenced by sex. Although male and female mice have similar Treg cell functioning, the capacity of effector cells to expand is better in males than in females post TCR stimulation, protecting male mice from autoimmune pathogenesis (Reddy et al. 2005).

Females consistently generate higher antibody responses and this has been documented in several infectious disease and vaccine trials (Fish 2008, Klein, Marriott, and Fish 2015). IgG antibody responses and class switching are influenced by female sex hormone by increasing their survival and activation (Lü et al. 2002, Grimaldi et al. 2002). Female mice mount higher antibody responses following sub-lethal dose of influenza and are subsequently better protected from

challenge (Klein, Hodgson, and Robinson 2012). Moreover, immunoglobulin responses are also affected by estrous cycle changes. Primate cervicovaginal secretions show highest IgG and IgA levels during menstruation and the lowest during ovulation (Beagley and Gockel 2003, Lü et al. 1999). Similar changes in IgG and IgA antigen secreting cells are also observed spleen and axillary, mesenteric, inguinal lymph nodes and PBMCs with highest levels during follicular phase (Lü et al. 1999, Yovel, Shakhar, and Ben-Eliyahu 2001). Meanwhile, elevated levels of testosterone are associated with a downregulation of influenza vaccine responses (Furman et al. 2014). Overall, consideration of sex and reproductive status in vaccine design may help alleviate adverse events reported in females and simultaneously improve vaccine efficacy in males.

Reproductive Senescence: (65 years and older)

Sex differences in longevity is well documented in humans with women having a greater lifespan than men by 6-8 years. Further, a similar trend is observed in other species of animals where the heterogametic sex tends to live longer. This observation eliminates anthropological/cultural reasons for female longevity but rather points to biological differences between the sexes (Candore et al. 2010, Tower and Arbeitman 2009, Jaspers et al. 2017). However, few studies consider sex differences in morbidity during the lifespan- whether females live longer but with sickness and disability. Therefore, an assessment of frailty and healthy aging in populations is an important milestone to comprehend sex differences in the elderly. A population-based Rotterdam Study developed a healthy aging score to assess age and sex differences in aging populations. A key finding was a lower healthy aging score in women indicating a higher disability, increased pain and worse mental health compared to aging men (Jaspers et al. 2017). Chronic diseases, on the other hand, were higher in aged men compared to aged women (Jaspers et al. 2017).

Sex differences in immunosenescence are still poorly understood. Aging causes a decline of sex steroid hormones, which in-turn may lead to loss of vigor and functioning of immune cells.

In females, about 80% of their estrogens are lost each year with the onset of menopause (Horstman et al. 2012). Contrary to this, andropause in males is more gradual. Males start to lose 1-3% of circulating testosterone around 35-40 years of age (Horstman et al. 2012). Apart from sex steroids, changes in the hypothalamic-pituitary-gonadal axis can affect other hormone levels as well. Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) levels also reduce (more drastically in females) and are implicated in development of co-morbidities like diabetes and cardiovascular diseases. Such a fall in GH and IGF-1 levels can have a profound impact on hematopoiesis, thymus functioning, autoimmunity and other inflammatory pathways (Smith 2010). Aging in women can affect XCI and women in their fifth decade show an increased degree of skewing during this X inactivation (Mengel-From et al. 2012). Similarly, in males, mosaicism is observed with aging through a loss of Y chromosome in blood cells which potentially disrupts immune surveillance and such a loss is commonly associated with neurodegenerative disorders (Forsberg 2017). Moreover, impaired mitochondrial DNA (mtDNA) through accumulation of mutations with aging is hypothesized to progressively increase oxidative damage (Barzilai et al. 2012, Tower 2015). Sex differences in aging mtDNA could further explain the increased telomere erosion in males- a decline in pangenomic telomere lengths with age (Möller et al. 2009). In addition, experiments in mice indicate age or sex related changes in microRNAs expression in the thymus which are involved in MAPK, PI3K-Akt and adipocytokine signaling pathways (Guo et al. 2017). Nevertheless, the impact of such changes in thymic involution and on sexual dimorphism in aging elderly is yet to be understood.

Sex-specific differences in phenotypes and subpopulations of cells are also observed in aging innate immune cells. For example, monocyte and leukocyte populations in aged males and females express different levels of receptors; males show higher CD38 expression while females show higher CD62L and CD115 expression, indicating differences in activation profiles and memory subpopulations. Aged males also have a higher proportion of non-classical monocytes

than aged females. However, the rate of occurrence of these changes is alike in both males and females. Sex differences among $CD14^{hi}CD16^{low}$ monocytes are also reported in aged individuals in which such differences in populations in monocytes and their descendants, can have an impact on phagocytosis, antigen presentation, lymphocyte activation and inflammatory responses, specifically ones that lead to chronic syndromes associated with aging (Al-Attar et al. 2016). $CD56^{dim}$ NK cells are more cytotoxic and more responsive to leukemic cells in aged females compared to aged males, which may explain the higher incidences of cancer in aged men compared to women in populations (Sieguel, Naishadham, and Jemal 2012).

Apart from changes in sex steroid levels, the steroidogenic capacity of immune cells can also be affected by aging. PBMCs from aged males show an increased expression of 17β-HSD5 activity that leads to a higher production of androstenedione from circulating dihydrotestosterone, a plausible compensatory mechanism (Hammer et al. 2005). However, whether similar mechanisms exist in PBMCs from females remains to be seen.

Transcriptional analysis in aging human PBMCs revealed several pro-inflammatory pathways that were affected only in aged females but not in aged males. Such pathways involved NF-κB signaling, NO synthesis and p38 MAPK signaling. Several sex-specific differences also arose in T cell activation pathways, indicating weaker T cell responses in female nonagenarians compared to their male counterparts (Marttila et al. 2013). Additionally, several cytokines show differential levels in blood circulation between the sexes. IL-15 is an important homeostatic cytokine in T, NK cell and memory responses and its pathway is significantly upregulated in aged females (Marttila et al. 2013). After menopause, there is a significant increase in pro-inflammatory cytokines IL-1, IL-6 and TNFα but a reduction in IFNγ are detected after menopause (Deguchi et al. 2001, Kumru, Godekmerdan, and Yılmaz 2004). Aging males, on the other hand, show an increase in soluble IL-6 receptor in serum and such an increase correlates to loss of testosterone with aging (Maggio et al. 2006). Loss of estrogen with menopause can impact

proliferation of B cells, significantly lowering the percentage of conventional B cells (B-2) in circulation (Kamada et al. 2001). Similarly, males experience a loss of activated B cells in circulation with aging (Inal et al. 2014). Absolute lymphocyte numbers in a study population showed a decrease with age in males but an increase with age in women. CD3+ T cells and naïve T cells reduced with age but to a much greater extent in males. CD4+ T cell counts increase with age in women but remain steady in males. Furthermore, the ratio of CD4/CD8 T cells increases significantly more in aged males than in aged females (Hirokawa et al. 2013, Strindhall et al. 2013). Importantly, the T cell proliferative capacity is preserved in women more than in men through the aging process which is an important factor to consider during vaccine design (Hirokawa et al. 2013). Other studies in mouse models focusing on gut microbiota reveal pronounced changes with aging in a sex-dependent manner. Loss of mucosal thickness and changes in microbial strains influenced T lymphocyte populations- specifically, an increase in systemic T_H17 responses with age (Elderman et al. 2017).

In conclusion, menopause and andropause, can lead to significant changes in lymphocyte subpopulations. Aging also affects microbiome, X-chromosome inactivation and loss of other housekeeping functions leading to an overall immunosuppression. However, such functions seem to be better preserved in aging females than aging males.

	 Dendritic Cells	 Monocytes & Macrophages	 Granulocytes	 Innate Lymphoid Cells	 Natural Killer cells	 B cells	 T cells
Sex differences in young adults	♀>♂ TLR7 activity H Type 1 IFN activity H ♂>♀ IL-10 production R,H	♀>♂ Activation M Phagocytic capacity M IL-10 production M M2 polarization M ♂>♀ TLR4 expression M Pro-inflammatory cytokine production M M1 polarization M	♀>♂ Phagocytic capacity M Neutrophil count M Nitric Oxide production post stimulation H,R,M ♂>♀ Neutrophil attractant chemokines R TLR9 expression M	♀>♂ Type 2 cytokine levels upon stimulation M ♂>♀ Type 2 ILC count H IL-13 production upon stimulation M	♂>♀ NK cell activity R ♀=♂ NK cell count H	♀>♂ B cell numbers H,M Antibody production H,M % switched memory B cells H	♀>♂ $CD4^+$ T cell count H,M $CD4^+/CD8^+$ T cell ratio H Activated T cell count M T cell proliferative capacity M Cytotoxic T cell activity H ♂>♀ $CD8^+$ T cell count M T_{reg} count M
Sex differences in aged adults	♀>♂ Nitric oxide synthesis H Mammalian family of mitogen-activated protein kinases (MAPK) signaling H,M IL-15 production H	♀>♂ CD62L, CD115 H expression ♂>♀ CD38 expression H Non-classical monocyte count H	ND	ND	♀>♂ NK cytotoxicity H Immunosurveillance H	♀>♂ Antibody production H Age-associated B cell count H,M	♀>♂ $CD3^+$ T cell count H $CD4^+$ T cell count P $CD4^+/CD8^+$ T cell ratio P T_H1 response M Naïve $CD8^+$ T effector memory cells P T cell proliferative capacity P,H ♂>♀ $CD8^+$ T cell count P

Table 1: Sex differences in innate and adaptive immune responses in young and aged individuals.

Vaccination is currently the primary and most effective method to prevent influenza disease. A meta-analysis of influenza vaccine studies between 2009-2016 estimates the seasonal influenza vaccine efficacy (IVE) against hospitalizations at 41% and monovalent pandemic vaccine showing highest efficacy at 72% in young adults (Rondy et al. 2017). However, most studies do not consider the sex effect when analyzing vaccine responses. Other human and small animal studies that did look at sex differences show higher vaccine responses in females (Klein, Hodgson, and Robinson 2012). In a trivalent influenza vaccine study, females develop higher hemagglutination inhibition (HAI) antibody titers (Engler et al. 2008). Reporting of injection site swelling and inflammation is also greater in females following receipt of TIV (Engler et al. 2008, Cook 2008). Among aged individuals, several studies report higher antibody titers, local and

systemic reactions at injection site in females (Falsey et al. 2009, Talaat et al. 2010, Hui et al. 2006, Cook et al. 2006).

To better understand the interaction between age and sex we endeavored to look at immune responses in younger vs. older humans and in young vs aged mice. In the mice, we also conducted a challenge to investigate whether age or sex had an effect on challenge outcome.

METHODS

Animals

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee (MO15H236). Adult (8-10 weeks) and aged (68-70 weeks) male and female C57BL/6J mice were purchased from Jackson Laboratories and housed at 5 animals per cage. The mice were housed under standard biosafety level 2 housing with food and water provided *ad libitum.* All animals were vaccinated with two doses of 30µg mouse-adapted influenza A virus ma2009 H1N1 (Hall et al. 2017) spaced 21 days apart. Retro-orbital bleeds were performed to collect blood at 1 and 2 weeks after boost. Three weeks after the boost, the mice were challenged with a mouse-adapted A/California/04/09 drift variant (ma2009dv) containing a K166Q mutation in HA sequence. This challenge was performed under anesthesia using a ketamine-xylazine cocktail and intranasal inoculation with 30µl 10^5 $TCID_{50.}$ The mice were then monitored for 2 weeks or euthanized at specific timepoints to collect blood and lung tissue (Figure 1)

Human Serum Samples

Human serum samples were obtained through a clinical trial by Chen et al., (Chen et al. 2012) from the Center for Excellence in Influenza Research & Surveillance (CEIRS) network. The subjects were vaccinated with two 30µg monovalent unadjuvanted split-virus H1N1 A/California/07/09 NYMC-X-179A strain manufactured by CSL Biotherapies. Subjects were stratified into two age groups (18-41 years and 65+ years). Serum samples were obtained before, 8 days and 21 days after vaccinations (Figure 2)

Anti-influenza IgG/IgG1/IgG2c ELISA

Enzyme-linked immunosorbent assay (ELISA) plates (Flat bottomed, Microlon 96-well high-binding plates manufactured by Greiner Bio-One) were coated with 100ng of purified virus protein in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing with wash buffer (1x PBS + 0.1% Tween-20), the plates were blocked with 10% dry milk (in PBS) solution and incubated for 1hr at 37°C. The blocking buffer was dumped, serially diluted serum was added and incubated at 37°C for 1hr. After washing three times with wash buffer, secondary antibody (IgG/IgG1/IgG2c) was added and incubated for 1hr at 37°C. The plates were washed three times with wash buffer and reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB) for 20 minutes. The reaction was stopped using 1N HCl. Absorbance was measured at 450nm using a plate reader. End cut-off is determined by calculating three times the background absorbance.

Anti-influenza avidity ELISA

Enzyme-linked immunosorbent assay (ELISA) plates (Flat bottomed, Microlon 96-well high-binding plates manufactured by Greiner Bio-One) were coated with 100ng of purified virus protein in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing with wash buffer (1x PBS + 0.1% Tween-20), the plates were blocked with 10% dry milk in PBS (or 10% FBS in PBS for human assay) solution and incubated for 1hr at 37°C. The blocking buffer was dumped, serum was plated in quadruplets at 1:60 dilution (in 5% FBS in PBS) and incubated for 1hr at 37°C. Ammonium thiocyanate (Sigma 221988-100G) at 2-3M concentration was added to the serum in duplicate while the other two receive only PBS. After exactly 15 minutes, the samples were washed eight times with buffer. IgG secondary antibody was added and incubated for 1hr at 37°C. The plates were washed three times with wash buffer and reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB) for 20 minutes. The reaction was stopped using 1N HCl. Absorbance was measured at 450nm using a plate reader and final ratios

were obtained by dividing normalizing samples with ammonium thiocyanate to corresponding ones without it.

Anti-influenza neutralizing antibody assay

Human serum samples were mixed with receptor destroying enzyme (RDE) manufactured by Denka Seiken Co. Ltd at 1:3 ratio and incubated overnight at 37°C. The mixtures were heated to 57°C for 30 minutes. Serum was serially diluted, mixed with $100TCID_{50}$ of virus (A/California/07/2009 or A/Michigan/45/2015) and incubated for 1 hour at room temperature. The mixtures were added to confluent Madin-Darby Canine Kidney (MDCK) cells, incubated at 37°C for 24 hours. The cells were washed with 1xPBS (containing Ca^{2+}&Mg^{2+}), fresh media was added and inoculated at 37°C for 3 days. 4% formaldehyde was used to fix the cells for 1-2 hours. The cells were stained with naphthol blue black overnight. The titer was calculated as the highest dilution that eliminated virus cytopathic effects in 50% of the wells (i.e., 2 out of 4 wells per dilution).

Mouse serum was heat inactivated at 56°C for 35 minutes. The serum was serially diluted, mixed with $100TCID_{50}$ of virus (ma2009 or ma2009d.v.), incubated for 1 hour at room temperature and added to confluent MDCK cells. After a 24hr incubation at 37°C, the cells were washed with 1xPBS (containing Ca^{2+}&Mg^{2+}), fresh media was added and inoculated at 32°C for 6 days. 4% formaldehyde was used to fix the cells for 1-2 hours. The cells were stained with naphthol blue black overnight. The titer was calculated as the highest dilution that eliminated virus cytopathic effects in 50% of the wells (i.e., 2 out of 4 wells per dilution).

$TCID_{50}$ virus quantification assay

Frozen lung samples were homogenized, centrifuged and supernatant was separated out. Log_{10} dilutions of homogenized lung are plated onto MDCK cells in six replicates. They are

incubated for 6 days at 32°C. The cells were then fixed with 4% formaldehyde, stained with naphthol blue black and titer was calculated using Reed-Muench method.

Pro-inflammatory human cytokine assay

The human serum samples were assayed in singlicate using MESO QuickPlex SQ120 kit was used to measure 10 proinflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α) were measured. For statistical analysis, any value below the lowest limit of detection was excluded.

Hemagglutinin Inhibition Assay

Mouse serum samples were heat inactivated at 56°C for 35 minutes, diluted with 100µl of 5% sodium citrate and sterile normal sodium chloride solution to obtain a final dilution of 1:20, incubated at 37°C for 30 minutes and cooled to room temperature. One drop (~50µl) of packed turkey red blood cells was added to the sample, centrifuged at 2000rpm for 10 minutes and supernatant was separated out. Hemagglutination assay (HA) was performed by serially diluting 25µl of virus across a round-bottom 96 well plate in 25µl of 0.01M PBS. 50µl of 0.5% RBC suspension was added to the virus dilutions and incubated at room temperature for 2 hours. The HA titer was determined by the highest dilution causing complete agglutination. For the hemagglutination inhibition assay (HAI), mouse serum was serially diluted across the plate, 25µl of 4 HA virus was added to each well and incubated at room temperature for 1 hour. 50µl of 0.5% RBC suspension was added and the mixture could agglutinate at room temperature for 2 hours. The HAI titer was calculated as the reciprocal of the highest dilution causing a complete inhibition of agglutination.

Statistics

Antibody responses, cytokine concentrations and viral titers were analyzed using a two-way ANOVA followed by post-hoc multiple comparisons using Sidak's multiple comparison test. Morbidity data (i.e., changes in body mass) was analyzed with a repeated measures MANOVA and post-hoc tests were performed correcting for multiple comparisons using Bonferroni-Dunn method. Survival following challenge was analyzed using Kaplan-Meyer curve and a Mantel-Cox log rank test. Correlation between groups was performed using Pearson r test. Mean differences were considered statistically significant if $p<0.05$.

RESULTS

Human females develop higher antibody titers post vaccination among young, but not aged, adult vaccinees

To test the hypothesis that sex differences in vaccine induced immunity is dependent on age, I compared cytokine and antibody responses among young (18-45 years) and aged (65+ years) adults prior to and after vaccination with an inactivated, monovalent A/California/2009 virus. Seasonal influenza vaccination has been shown to induce proinflammatory responses immediately after receipt of the vaccine, which can be dependent on both the age and sex of the vaccinee (Furman et al. 2014). To test the hypothesis that sex- and age-associated differences in proinflammatory immune responses occur following receipt of the monovalent 2009 H1N1 vaccine, concentrations of IFNγ, TNFα, IL-10, IL-6, and IL-8 were measured in serum samples collected prior to and 8 days post-vaccination. All cytokines were detected in serum and increased following vaccination; IL-6, however, was the only cytokine that was differentially produced based on the sex and age of the vaccinees. Specifically, young adult females had significantly higher concentrations of IL-6 post-vaccination than either young adult males or aged adult females (**Figure 3a**, $p<0.05$). Because IL-6 is crucial for germinal center formation, antibody production and class switching (Kopf et al. 1998) this observation suggests that young adult females should also have higher antibody responses than either young adult males or aged adult females. Further, a strong correlation was observed between fold change in IL-6 and neutralizing antibody responses (**Figure 3b**, $r^2= 0.2294$, $p<0.05$)

To measure influenza virus vaccine efficacy, either HAI titers or neutralizing antibody titers can be measured. HAI titers reflect the antibodies that prevent viral attachment to red blood cells (hemagglutination) while neutralization assay allows determination of antibodies that can

block viruses from infecting cells (Hancock et al. 2009). Thus, neutralizing antibody titers are presumed to be a superior measure of vaccine efficacy (Co et al. 2012, Fox et al. 2015). In contrast to previous studies that measured HAI titers following receipt of a seasonal influenza TIV and reported sex differences in HAI titers among both young adult and aged adult vaccinees (Engler et al. 2008, Cook 2008), no sex or age differences were observed in HAI titers following receipt of the monovalent 2009 H1N1 vaccine (**Figure 3c**). Neutralizing antibody titers were higher in young adult females compared to young adult males (**Figures 3d**, $p<0.05$). Young adult females also had higher neutralizing antibody titers than aged adult females. No sex differences in neutralizing antibody titers were observed among aged adults.

In addition to the quantity or titer of antibody, the quality of antibody or the strength of binding can be assessed for vaccine efficacy (Verma et al. 2012). To assess the quality of in antibody binding strength, anti-influenza virus IgG avidity and cross-neutralization were analyzed. Sex differences in IgG avidity were not observed among young adult vaccines (**Figure 3e**). In contrast, among aged individuals, males showed higher avidity antibodies compared to aged females (Figure 3e, $p<0.05$). Aged males also had higher IgG avidity than young males. (**Figure 3e**, $p<0.05$). The cross-reactivity of neutralizing antibodies was assessed by measuring the neutralizing activity against the 2017 H1N1 vaccine strain A/Michigan/2015. Overall, young adults showed higher cross-neutralization than aged adults, pointing to a potential inability by aged individuals to mount an adequate response to new strains (**Figure 3f**, $p<0.05$). Sex differences in cross-neutralization, however, were not observed among either young or aged adults (**Figure 3f**). Taken together, these data also indicate that prior exposure to influenza and vaccine history play and important role in determining avidity or cross-neutralization activity.

Among mice, sex differences in antibody quantity and quality are apparent among young but not aged adults following vaccination

Naïve young and aged mice were vaccinated with two doses of mouse adapted 2009 H1N1 vaccine and antibody titers were measured. Consistent with previous studies, young adult females developed higher HAI titers to the 2009 H1N1 vaccine than either young adult males or aged adult females **(Figures 4a**, $p<0.05$**)** (Živković et al. 2015). Young adult female mice also had higher neutralizing antibody titers to 2009 H1N1 than either young adult males or aged adult females (**Figure 4b**, $p<0.05$). In contrast, no age associated changes in either HAI or neutralizing antibody titers to 2009 H1N1 were observed among male mice (**Figures 4a and 4b**).

To assess the quality of antibody responses in mice, cross-neutralizing antibody titers, anti-2009 H1N1 IgG and IgG isotypes, as well as IgG avidity were measured. Young adult female mice had higher cross-neutralizing antibody titers against a 2009 H1N1 drift variant virus than either young adult males or aged adult females (**Figure 4c**, $p<005$). In contrast, cross-neutralizing antibody titers did not differ between young and aged adult male mice (**Figure 4c**). In contrast to humans (**Figure 3e**), young adult female mice had a greater IgG avidity index than either young adult male or aged adult female mice (**Figure 4d**, $p<0.05$). Total anti-2009 H1N1 IgG titers were also higher in young adult females than either young adult male or aged adult female mice (Figure 5a, $p<0.05$), with no significant differences observed between young adult and aged adult male mice.

IgG isotypes differ in the avidity to influenza viruses, with IgG2c being of greater avidity than IgG1 and being associated with better vaccine efficacy (Vidarsson, Dekkers, and Rispens 2014, Huber et al. 2006). Among the IgG subtypes, IgG2c is shown to be efficient at complement fixation and is necessary for viral clearance (Huber et al. 2001). Overall, young adult mice had higher anti-2009 H1N1 IgG1 titers than aged adult mice (**Figure 5b**, $p<0.05$), but sex differences were not observed. In contrast, not only did young adult mice have higher anti-2009 H1N1 IgG2c titers than aged adult mice, titers were significantly higher among young adult females than either young adult males or aged adult females (**Figure 5c**, $p<0.05$). These data

suggest that vaccine efficacy may be greater for young adult females than either young adult males or aged adult females.

Sex differences in protection against influenza virus challenge are apparent among young but not aged adult mice

Utilizing a mouse model allows for direct testing of vaccine efficacy though influenza virus challenge of vaccinated mice. In this study, vaccinated mice were challenged with a drift variant 2009 H1N1 virus (2009 H1N1 dv) to measure effectiveness of the vaccine for protection against infection. Overall, young adult mice that were vaccinated with 2009 H1N1 were better protected against challenge with the 2009 H1N1 drift variant virus, experiencing less morbidity and mortality and clearing influenza virus from their lungs faster than aged adult mice (**Figure 5d-5f**, $p<0.05$ in each case). Sex differences in protection against influenza virus challenge were also observed. Specifically, vaccinated young adult females were better protected following influenza virus challenge, losing significantly less body mass than either young adult males or aged adult females **(Figure 5d**, $p<0.05$**)**. Further, vaccinated young adult females had lower lung viral titers three days after challenge than either young adult males or aged adult females **(Figure 5f)**. Overall, vaccinated young adult males and females cleared virus from their lungs faster than aged adult mice **(Figure 5f)**. Overall, these data suggest that the high neutralizing antibody titers correlate to protection in young female mice. The vaccine efficacy is lower in young male and aged mice.

Neutralizing and HAI titers are correlated among vaccinated mice

Several studies claim that HAI and neutralizing antibody titers are correlated (Cheng et al. 2012). One assumption is that this is true among all individuals, regardless of the sex, age, or

immune status of the individual. To test this hypothesis, I evaluated the association between HAI titers and neutralizing antibody titers following immunization with the 2009 H1N1 vaccine in humans as well as mice. Among humans, HAI titers and neutralizing antibody titers were not correlated within any of the individual groups (**Figure 6a**). However, after pooling all the groups together, a significant correlation was observed (p=0.019), suggesting that our sample sizes are not sufficient to detect meaningful associations between these measures in humans. Among mice, where sample sizes were sufficient to observe meaningful differences, HAI and neutralizing titers correlated among both young adult and aged adult male and female mice **(Figure 6b**, p<0.05**)**. Taken together, this potentially indicates neutralizing antibody test as a more sensitive assay to study sex and age effects on antibody responses.

Figure 1: Mouse experimental design

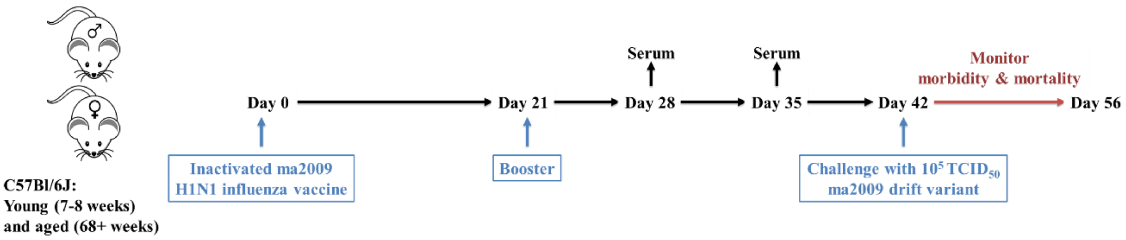


Figure 2: Human study design

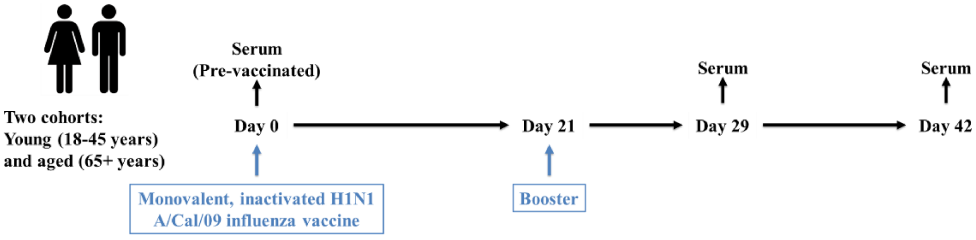


Figure 3: Human serologic responses following immunization with the 2009 H1N1 monovalent vaccine

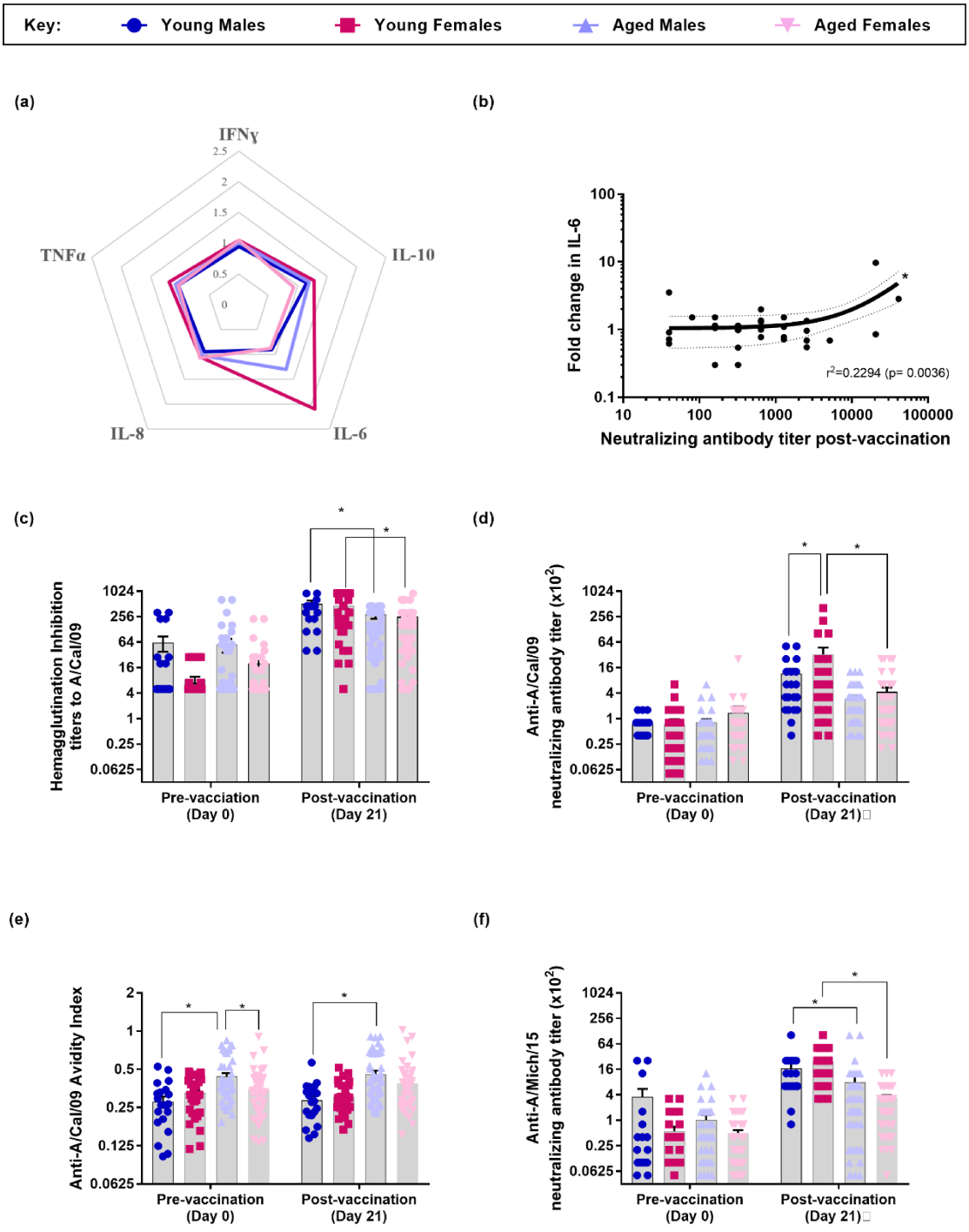


Figure 4: Mouse serologic responses following immunization with a 2009 H1N1 vaccine

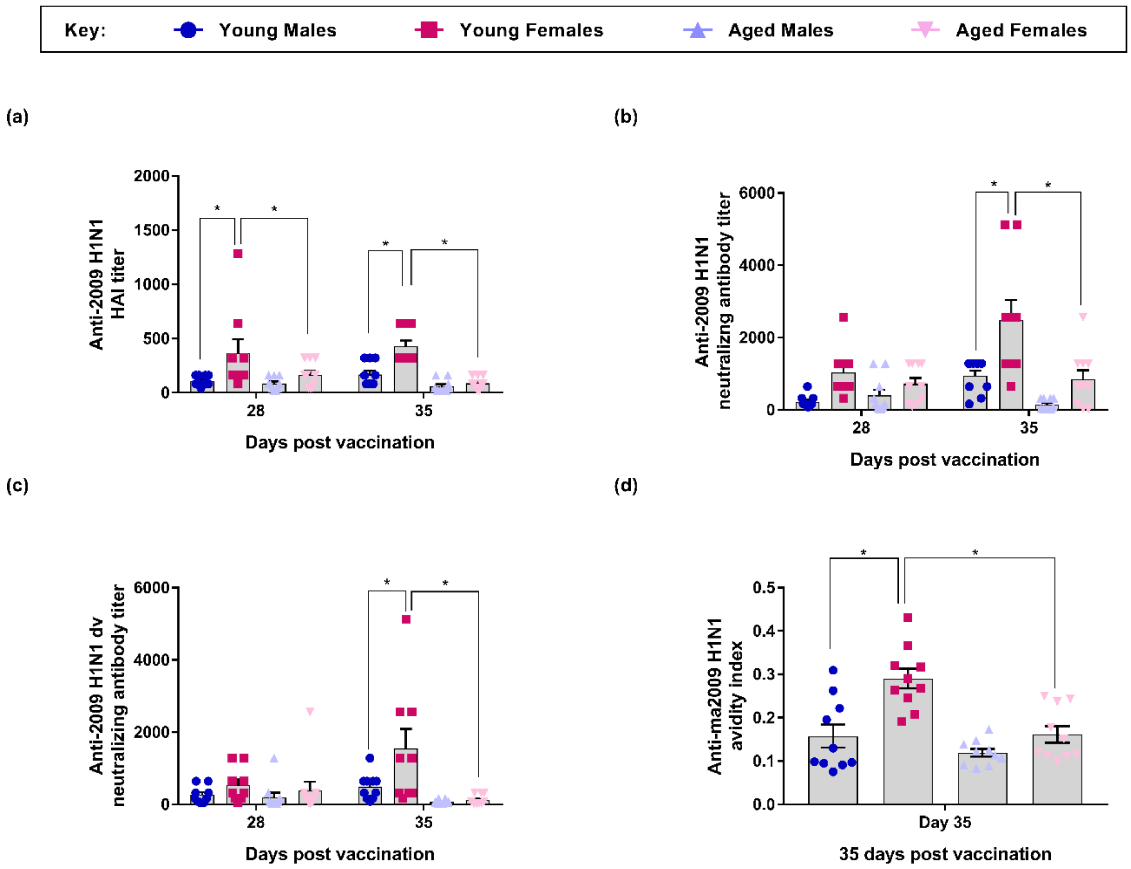


Figure 5: Mouse anti-2009 H1N1 IgG responses and protection from influenza virus challenge

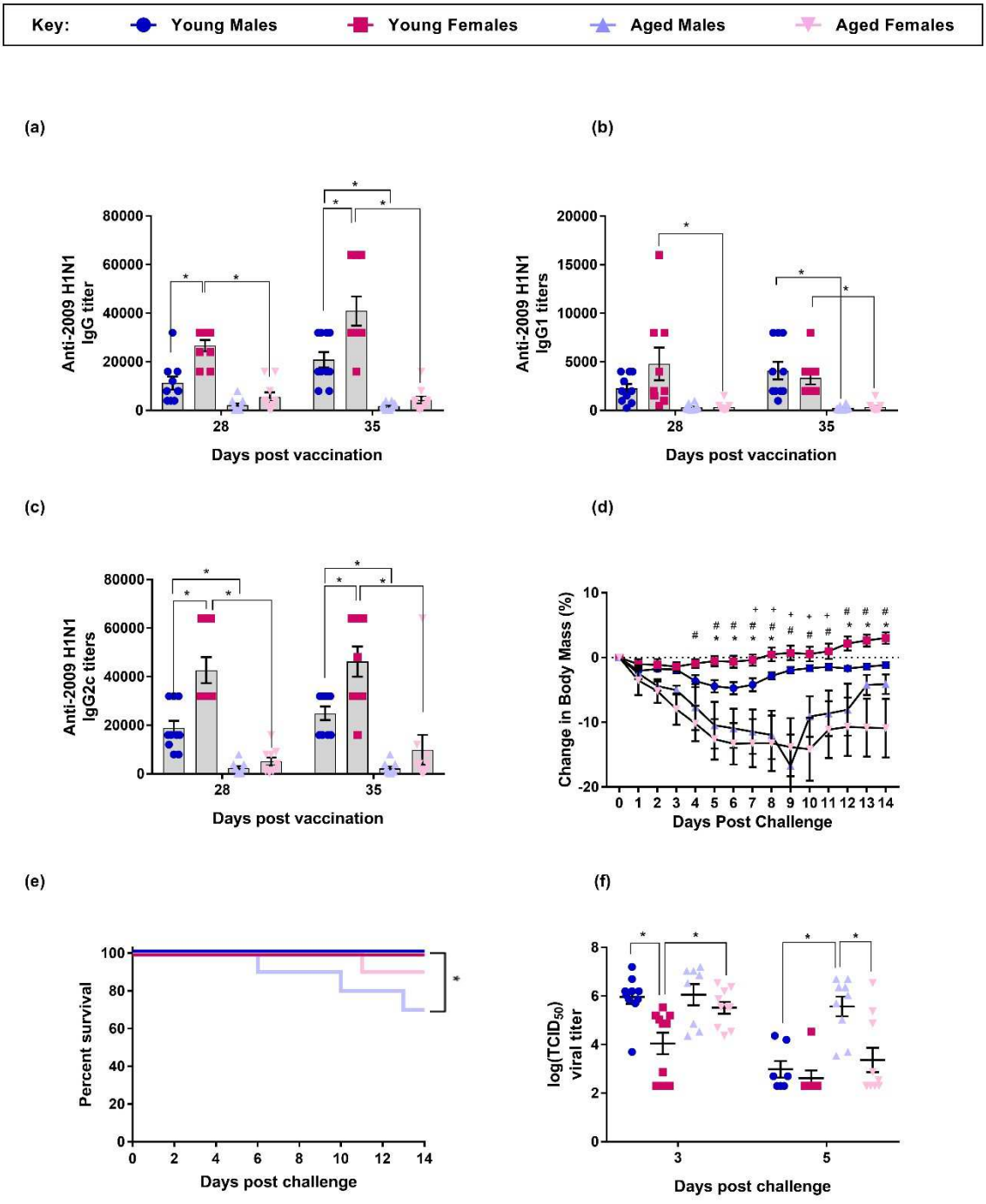
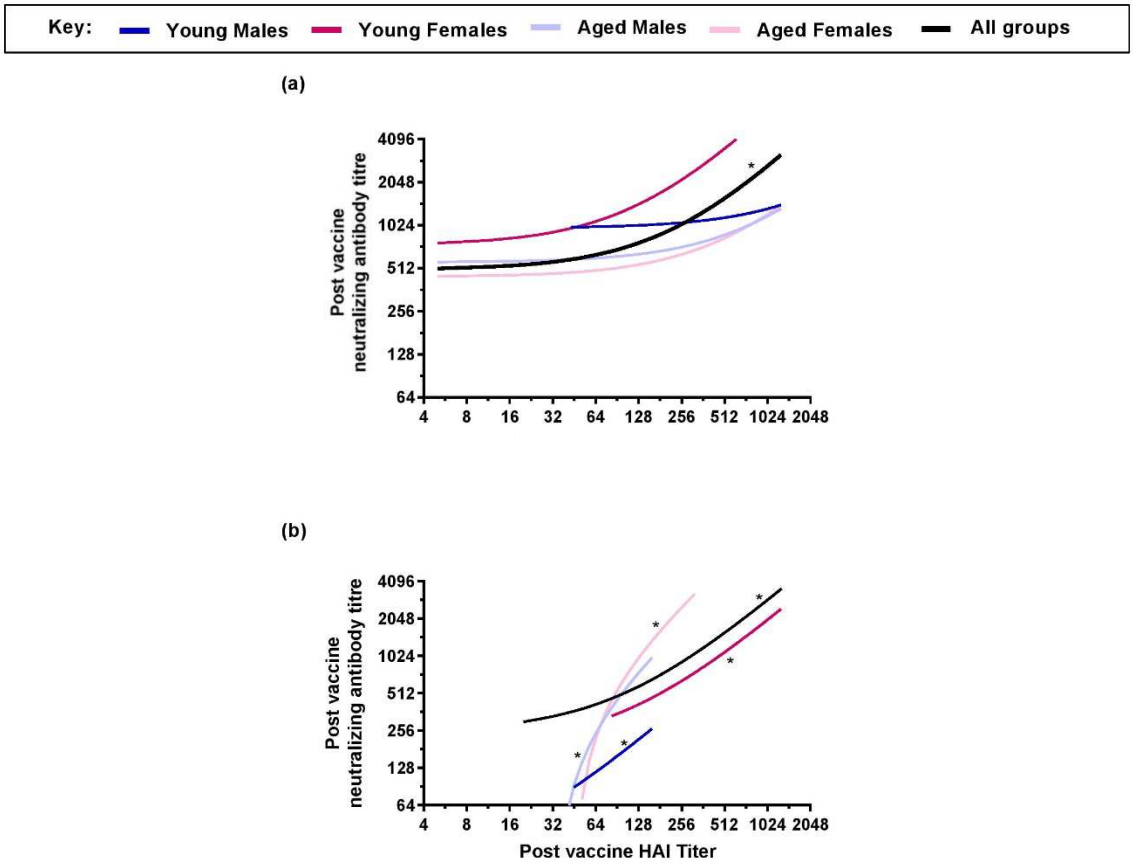


Figure 6: Comparison between neutralizing and hemagglutination inhibition antibody titers in humans (a) and mice (b)



DISCUSSION

Seasonal and pandemic influenza outbreaks have a massive public health impact worldwide. In the United States alone, the CDC estimates 9.2-35.6 million cases of illnesses and 12000-56000 mortalities annually since 2010. Elderly individuals with a higher burden of chronic conditions are at a greater risk of hospitalizations (Mauskopf et al. 2013). In 2017, 13% of the world population is above 60 years of age. The United Nations predicts rapid aging (faster growth than any other younger age groups) in all regions of the world and such a trend is estimated to continue with 21% of population made up of elderly individuals by 2050 (United Nations 2017). Such an increase in aging population poses significant public health challenges, especially as they tend to suffer from more severe and frequent infections compared to younger individuals. Influenza particularly has a major impact on aging populations with ~90% of mortalities due to influenza are in individuals above 65 years of age. They represent a majority of hospitalizations due to influenza like illnesses and have a major socio-economic impact (Aspinall et al. 2007).

A key strategy to reduce adverse influenza outcomes is to provide effective immunizations. Both direct and indirect impacts of influenza vaccinations have been studied. Protection from infection can be considered a direct impact of vaccination while blocking of transmission is the indirect impact (Dorratoltaj et al. 2017). For example, in a multi-center case controlled study analyzing direct impact of influenza vaccinations, hospitalized patients with influenza showed 55% lowered odds of severity of disease if they were vaccinated for any of the three previous seasons. Their odds of admission to ICU and death reduced by 65% and 56% by vaccination to any one of the past three seasons (Casado et al. 2018).

Economically, an outbreak could lead to a $25.4 billion loss of U.S. GDP (Prager, Wei, and Rose 2017). Studies show vaccine interventions are cost-beneficial and have high return on investment

and net benefits on the economy among both high risk and non-high risk groups (Dorratoltaj et al. 2017).

In this study, we analyze vaccine responses using a "sex and age" lens. Analysis of cytokine changes in human serology is useful in predicting the immunogenicity of vaccinations. Host factors and baseline serum cytokine levels have been shown to correlate with vaccine responses in previous studies (Krakauer and Russo 2001). In our study, serum IL-6 significantly increased with vaccination in young adult females. IL-6 is shown to promote T-follicular helper cell and activated B cell differentiation (Tanaka, Narazaki, and Kishimoto 2014). Although sex differences in serum IL-6 has not been investigated in the context of influenza vaccinations, previous studies have shown sex differences in stressor-evoked IL-6 and C-reactive protein levels (CRP). Females showed a greater increase in IL-6 from baseline compared to males post stress tasks (Lockwood et al. 2016).

Human females of reproductive ages generated higher neutralizing antibody responses towards the vaccine strain A/Cal/09. Such a sex difference was not observed using the HAI test **(Figures 3c and d)**. A comparison between HAI and neutralizing antibodies was performed and showed no correlation amongst the groups **(Figure 6a)**. Due to the higher variability in human serology, analyzing all groups together showed a significant correlation but not within individual groups. For several decades, HAI antibodies have been shown to correlate to protection and neutralization *in vitro* and in animal models (Hobson et al. 1972, Couch and Kasel 1983). However, such studies failed to take into account the different strains of influenza, types of vaccines and route of infection/immunization (Fox et al. 2015). Further, correlations between HAI and neutralizing antibodies may vary depending upon pre-existing immunity and exposure to previous circulating strains. HAI assays also lack the sensitivity and functional significance provided by neutralizing assays (Benne et al. 1998, Zhu et al. 2011). Furthermore, several broadly neutralizing antibodies may not exhibit any HAI activity (Fox et al. 2015). It is also important to note the presence of

several factors in serum and plasma that interfere with HAI and neutralizing assays (Fox et al. 2015). Therefore, inter and intra-laboratory variability may exist depending on the anti-coagulants and inactivation techniques used (Benne et al. 1998, Truelove et al. 2016). Understanding antibody responses after influenza vaccinations and whether they correlate to protection is crucial. HAI assays measure antibodies that bind to hemagglutinin receptor site and block its interaction with sialic acid on erythrocytes. On the other hand, neutralization assay and the complement dependent lytic (CDL) assay are functional tests that detect antibodies that neutralize the virus or lyse virus-infected cells. Such assays could better correlate to protection after infections. Clinical studies are therefore, required to understand the role of HAI, neutralizing and CDL antibodies after vaccinations, especially in populations that received influenza vaccines previously (Co et al. 2012).

Thymic involution and increase in proportion of memory populations with aging render aged individuals unable to mount responses to novel strains and this was demonstrated in their cross-reactive neutralizing antibody responses to A/Michigan/15 strain **(Figure 3f)**. This points to impaired somatic hypermutation and class switching processes in aged germinal centers. Such a reduction B cell diversity with age has been observed in previous studies (Scholz et al. 2013).

Avidity is the measure of functional affinity or the overall strength of antibody-antigen binding. None of the groups showed an increase or decrease of avidity with vaccinations. Although aged males produced higher avidity antibodies, this was observed in pre-vaccination titers as well, suggesting a previous exposure to the virus among the study group. Further, such high avidity, non-neutralizing antibodies have been associated with more severe influenza in previous studies. (To et al. 2012)

Overall, we see reduced antibody responses in aged individuals be it to vaccine or novel strains. Further investigation is needed to better understand this age-related decline in vaccine responses.

Whether this effect can be countered by targeting plasmablast production, antibody class switching functions or memory cell populations should be addressed.

This study also sheds light on the differences seen in vaccine responses when comparing animal and human studies. Translating a human study to a naïve mouse model, we can demonstrate a biological sex and age effect without the confounding effect of gender behaviors and pre-existing immunity. Mouse models of influenza have several other advantages including their ready availability, low costs, easy housing and availability of numerous knockouts, knockins, transgenic strains that allow us to study influenza pathogenesis. However, a major limitation is the inability to recreate the complexity of previous infections and therefore inefficient translation to humans. Such differences are clearly observed in this study where HI and neutralizing antibody titers correlate strongly in mouse model but not in humans among the four age and sex groups **(Figures 6a and b)**.

Sex differences were seen in young mice in antibody responses to vaccination and morbidity following challenge but no such sex differences were observed in aged animals **(Figures 4 and 5)**. Young females generated significantly higher neutralizing antibody titers to ma2009 and ma2009 d.v. after vaccination. This is consistent with several previous studies that reported sex differences in antibody titers with vaccinations (Lorenzo et al. 2011). A similar pattern is observed with IgG and IgG subtype antibody titers were young females consistently developed high antibody titers compared to age-matched males or aged females. The exact mechanism for such an increase in antibody responses is yet to be grasped but sex steroid hormones have been implicated in playing a major role in modulating immune responses. Enhancement by estradiol or suppressive effect by testosterone on B cells could together mediate these sex differences in young adults (Klein 2000, Furman et al. 2014). Further, TLR7 in females is shown to escape X chromosome inactivation and therefore they could be more sensitive to ssRNA viruses, generating a more robust immune response compared to males (Souyris et al. 2018). Although

there is evidence of changes in XCI with aging, whether this is seen in regard to TLR7 is yet to be investigated (Mengel-From et al. 2012).

Upon challenging vaccinated mice, young females were better protected and thus higher neutralizing antibody titers correlate with lower morbidity after challenge **(Figure 5d)**. Vaccine efficacy against disease was lower in young males compared to young females or aged males. Aged males and females showed equally low protection after challenge with high morbidity and mortality **(Figures 5d and e)**. Protection against challenge can be considered primarily to be meadiated by antibodies. However, after infection, the severity and outcome of infection is mediated by cellular immune responses (Thomas et al. 2006).

Lung viral titers were low in young females compared to young males or aged females three days after challenge which also points to protection due to high antibody titers. Young males despite having high viral titers after three days could reduce the infection within five days, pointing to protection by cell-mediated immunity **(Figure 5f)**. Aged males were unable to clear virus and showed high titers five days after challenge. Such a viral persistence can point to impaired T cell responses in aged males as compared to aged females. As indicated by other studies, immunological pathways seem to be better conserved in aged females than aged males (Marttila et al. 2013).

Sub-optimal effectiveness of influenza vaccines in protection from disease are reported yearly leading to increased hospitalizations due to severe disease (Haq and McElhaney 2014). From this study, we show that inactivated influenza vaccine is sufficient to protect female mice from morbidity. However, the vaccine fails to sufficiently protect young males, aged females or aged males. Vaccines that induce stronger T cell responses may help improve vaccine efficacy in young males.

Healthy aging:

Healthy aging is aging with little or no disabilities in physical or cognitive functioning. This can be achieved by decreasing morbidity of diseases, minimizing disabilities and enhancing quality of life. There is a strong correlation between infectious diseases and disability in the elderly. Individuals with greater disability are more likely to contact infections and individuals more prone to infectious diseases are more likely to show high functional decline (Maggi 2010). Currently, vaccination is a simple and effective tool that can be used to promote healthy aging. One great example is the herpes zoster (shingles) vaccine where vaccination greatly improves the quality of life, reduces pain and complications in elderly populations (Maggi 2010). A similar positive effect can be observed by influenza immunization strategies (Casado et al. 2018). However, the low efficacy of seasonal influenza vaccinations poses a significant challenge and tailoring influenza vaccines based on sex and age could help improve vaccine responses. Redesigning existing vaccines by changing vaccination schedules, dosages, adjuvants, routes of immunization are some strategies that need to be tested specifically for elderly populations. Oil in water adjuvants (MF59, AS03) have been shown to improve influenza vaccine responses in the elderly (Boraschi et al. 2013). Other strategies such as reducing lipid accumulation (or BMI), hormone therapies can be studied to reduce the effects of immunosenescence. Adult influenza vaccinations are crucial in the efforts to promote healthy aging and a better understanding of sex and immunosenescence is the key to making such vaccinations.

FIGURE AND TABLE LEGENDS

- **Table 1: Sex differences in innate and adaptive immune responses among young and aged individuals**

  Summarized data from human (H), rats (R) non-human primate (P) and mouse (M) models. ND= not determined.

- **Figure 1: Mouse experimental design**

  Young and aged C57Bl/6J mice were administered two vaccinated with two doses of 30µg mouse-adapted influenza A virus ma2009 H1N1 (Hall et al. 2017) spaced 21 days apart. Retro-orbital bleeds were performed to collect blood at 1 and 2 weeks after boost. Three weeks after the boost, the mice were challenged with a mouse-adapted A/California/04/09 drift variant (ma2009dv) containing a K166Q mutation in HA sequence with an intranasal inoculation with 30µl 105 TCID50. The mice were then monitored for 2 weeks or euthanized at 3 and 5 days post challenge to collect blood and lung tissue (n=8-15/group)

- **Figure 2: Human study design**

  Young and aged male and female were vaccinated with two 30µg monovalent H1N1 A/California/09 strain. Serum samples were obtained before, 8 days and 21 days after vaccinations (n= 20-48/group)

- **Figure 3: Human serologic responses post vaccinations**

  Adults were administered inactivated, monovalent A/Cal/09 H1N1 vaccinations and serum cytokine fold change (a) correlation between IL-6 and neutralizing antibody responses were measured prior to and 8 days post vaccinations (b). Hemagglutination inhibition titers (c), neutralizing antibody titers (d), antibody avidity (e) and cross-

reactive neutralizing antibody titers to A/Mich/15 (2017 vaccine strain) (f) were measured prior to and 21 days post vaccinations. Data represents mean ± standard error of mean (n=20-48/group) and significant differences between groups are denoted by asterisks (*)

- **Figure 4: Mouse serologic responses post vaccinations**

  Mice were vaccinated with mouse-adapted 2009 H1N1, inactivated virus and hemagglutination inhibition titers (a), neutralizing antibody titers to vaccine (2009 H1N1) (b), antibody avidity (c) and cross-reactive neutralizing antibody titers to drift variant virus (2009 H1N1 dv) (d) were measured 28 and 35 days post vaccinations. Data represents mean ± standard error of mean from two different replications (n=8-10/group) and significant differences between groups are denoted by asterisks (*)

- **Figure 5: Mouse IgG responses and post challenge analysis**

  IgG (a), IgG1 (b) and IgG2c (c) antibody responses were measured 28 and 35 days after receipt of ma2009 vaccinations; loss of body mass was measured as a correlate of morbidity (d) and mortality (e) for 14 weeks after challenge with mouse adapted 2009 H1N1 drift variant virus; viral titers in lung homogenates 3 and 5 days after challenge (f) were measured (*$p<0.05$). Data represents means ± standard error of mean from two different replications (n=8-15/group) and significant differences between young males and females are denoted by asterisks (*); between young and aged females by pound sign (#) and between young and aged males by plus sign (+) in the morbidity data.

- **Figure 6: Comparison between neutralizing and hemagglutination inhibition antibody titers**

  Neutralizing and HAI titers post vaccinations are compared among 21 days post-vaccine titers in humans (n=20-48/group) (a) and 28 days post vaccine titers in mice (n=8-15/group) (b). Data represents linear regression with significant correlation indicated by asterisks (*).

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CURRICULUM VITIATE

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EDUCATION

May 2018 **Master of Science**
Department of Molecular Microbiology & Immunology
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- GPA- 3.95/4.00
- Master's Thesis: "Effects of sex and age on vaccine-induced immunity and protection"

June 2016 **Bachelor of Technology**
Department of Biotechnology
National Institute of Technology, Calicut, India

- GPA- 9.32/10
- Bachelor's Thesis: "Identification of endophytes involved in Zerumbone production in *Zingiber zerumbet* extract"

RESEARCH EXPERIENCE

Graduate Researcher
2016-2017
Laboratory of Dr. Sabra Klein, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- Evaluated age & sex differences in the immunological responses to influenza vaccine in humans
- Evaluated age and sex differences in influenza vaccine efficacy in a murine model
- Analyzed human serological responses to circulating influenza viruses through active influenza surveillance efforts

Undergraduate Researcher
2015
Laboratory of Dr. Brian Christie, University of Victoria, British Columbia, Canada

- Analyzed the effect of chronic running on hippocampal neurogenesis in Fragile X syndrome mice.
- Performed immunohistochemistry and behavioral analysis to document the extent of hippocampal neurogenesis
- Performed immunohistochemistry to analyze the rescue of fragile X mice via neurogenesis after minocycline treatment

Undergraduate Researcher
2013-2014
Laboratory of Dr. Suchitra T.V, National Institute of Technology, Calicut, India

- Performed qualitative and quantitative examination on bacteria isolated from diabetic foot ulcers
- Conducted various biochemical assays to examine enzymatic activity from bacteria associated with diabetic foot ulcers

Undergraduate Researcher
2015-2016
Laboratory of Dr. Aswati Nair, National Institute of Technology, Calicut, India

- Screening for endophytes involved in the production of chemotherapeutic compound Zerumbone from *Zingiber zerumbet* isolates.
- Performed chromatography techniques to extract endophytes producing bioactive compounds

PUBLICATIONS

- Gubbels Bupp M., **Potluri. T**., & Klein, S.L. *Sex hormones and aging of the immune system.* Invited review for Frontiers in Immunology. In review.
- **Potluri, T**., Engle, K., Fink, A.L., vom Steeg, L. G., & Klein, S. L. *Sex reporting in preclinical microbiological and immunological research*. 10.1128/mBio.01868-1
- Nampoothiri, S.S., **Potluri, T**., Subramanian, H. and Krishnamurthy, R.G., 2016. *Rodent Gymnastics: Neurobehavioral Assays in Ischemic Stroke*. Molecular Neurobiology, pp.1-12.
- Mathew, S.M., Ravisanker, V., **Potluri, T**. and Suchithra, T.V., 2015. *Delayed Diabetic Wound Healing: A focus on bacterial proteases in chronic wound and foot ulcer*. International Journal of Current Research and Review, *7*(12), p.36.

POSTER PRESENTATIONS

- *"Effects of sex and age on vaccine-induced immunity and protection".*
  Authors: Tanvi Potluri, Ashley L. Fink and Sabra L. Klein
  11th Annual Johns Hopkins Women's Health Research Symposium, Baltimore, MD. May 2018

- *"Effects of sex and age on vaccine-induced immunity and protection".*
  Authors: Tanvi Potluri, Ashley L. Fink and Sabra L. Klein
  Vaccine Day, Johns Hopkins Vaccine Initiative, Baltimore, MD. April 2018

- *"Effects of sex and age on vaccine-induced immunity and protection".*
  Authors: Tanvi Potluri, Ashley L. Fink and Sabra L. Klein
  Delta Omega Poster Competition, Baltimore, MD. March 2018.

- *"Sex and age differences in antibody profiles following influenza vaccinations"*
  Authors: Tanvi Potluri, Ashley L. Fink and Sabra L. Klein
  Annual Centers of Excellence for Influenza Research & Surveillance Conference, Atlanta, GA. July 2017.

- *"Sex and age differences in antibody profiles following influenza vaccinations"*
  Authors: Tanvi Potluri, Ashley L. Fink and Sabra L. Klein
  Molecular Microbiology & Immunology Department Retreat, Johns Hopkins University, Baltimore, MD. August 2017.

FELLOWSHIPS/AWARDS

Mitacs Globalink Research Fellowship Award
2015
- Research fellowship awarded by Mitacs Organization, Canada

University Gold Medal Recipient
2016
- National Institute of Technology, Calicut for highest standing in undergraduate Biotechnology

PROFESSIONAL EXPERIENCE

Ad-hoc Reviewer 2016- Present
- Clinical & Vaccine Immunology
- British Medical Journal

Administrative Coordinator 2017- Present
Johns Hopkins Center for Women's Health, Sex and Gender Research, Baltimore, MD
- Organized annual symposiums and networking events
- Coordinated the activities of the center

Graduate Assistant 2016- 2018
Insectary Core Facility, Johns Hopkins Malaria Research Institute, Baltimore, MD
- Part-time work maintaining mosquito larval trays

Teaching Assistant for Public Health Perspectives in Research course
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2017- Present
- Evaluated assignments and organized classes for academic term 2017-18